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Full Length Research Paper

Identification of the CagM's location in cytomembrane of *Helicobacter pylori*

Honglei Ding[1, 2, 3], Tao Liu[2], Linping Huang[2], Weijun Zhang[2], Ping Luo[2], Qinghua Xie[2], Zhen Liu[2], Gang Guo[2], Xuhu Mao[2]* and Quanming Zou[1,2]

[1]Institute of Sericulture and System Biology, Southwest University, Chongqing 400715, China.
[2]Department of Clinical Microbiology and Immunology, Faculty of Medical Laboratory Science, Third Military Medical University and National Engineering Technological Research Center of Immunological Biologicals, Chongqing 400038, China.
[3]Chongqing Productivity Council, Chongqing, China.

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Helicobacter pylori **infect nearly half of the world's population and are associated with a spectrum of gastric maladies. The cytotoxin-associated gene pathogenicity island (*cag* PAI) encoding a type IV secretion system (T4SS) has been implicated in a series of host responses during infection. The *cag* PAI contains about 28 - 30 open-reading frames, for most of which the exact function is not well characterized or totally unknown and a delivered effector, CagA that becomes tyrosine phosphorylated upon delivery into host cells and initiates the changes in cell signaling. Here, we cloned one such *cag* PAI protein, CagM, which is encoded by the gene *HP0537* from *H. pylori* strain 26695 and expressed the gene in *Escherichia coli* M15. 6xHis-tagged CagM protein was purified with one-step Ni-NTA affinity column chromatography. The IgG antibody against CagM was produced by immuning rabbit and purified by protein A sepharose chromatography, finally, we showed that CagM protein localized to the bacterial inner and outer membrane and the conclusion was consistent to some bioinformatics result.**

Key words: *Helicobacter pylori*, CagM, type IV secretion system, expression, fractionation.

INTRODUCTION

Helicobacter pylori, a microaerophilic, gram-negative bacterium that efficiently colonizes in the stomach of about half of the world's population, is one of the most common infectious agents and can be considered as a prototype for microbial persistence (Blaser and Atherton, 2004). A clinical spectrum of illnesses are associate with this risky factor, including peptic ulcer disease, chronic gastritis, mucosa-associated lymphoid tissue lymphoma and adenocarcinoma (Montecucco and Rappuoli, 2001; Peek and Blaser, 2002). Several bacterial factors that are thought to contribute to the process of infection and colonization of the gastric epithelium, such as urease, the vacuolating toxin Vac A, ferric uptake regulator (Fur), and various inflammatory processes related to the risk of diseases that allow the bacterium to elude the host immune response (Blaser and Atherton, 2004; Guo et al., 2008; Oleastro et al., 2006; Rieder et al., 2005). Also, severe diseases in infected patients are often associated with strains of *H. pylori* harboring an intact cytotoxin-associated gene pathogenicity island (*cag* PAI) (Bourzac and Guillemin, 2005; Censini et al., 1996).

The *cag* PAI of *H. pylori* is a 40 kb genomic insert region that is comprised of 28 to 30 genes (Akopyants et al., 1998; Censini et al., 1996). A portion of these genes, encode a type IV secretion system (T4SS), are respon- sible for the injection of the only known effector molecule, CagA, into host cell (Censini et al., 1996). CagA translocation induces several consequences that result in the scattering and elongation of infected host cells in cell culture, resembling those of malignant cellular trans- formation (Backert et al., 2001; Püls et al., 2002). Then, the host cell is tyrosine-phosphorylated and subsequently induces

*Corresponding author. E-mail: maoxuhu@163.com, mxh95xy@mail.tmmu.com.cn. Tel: 86-23-68752315. Fax: 86-23-68752315.

cytoskeletal rearrangements that either require, or are independent of tyrosine phosphorylation (Backert and Selbach, 2008; Bourzac and Guillemin, 2005). Another cellular response caused by the *cag* secretion system is the production and secretion of interleukin-8 (IL-8) by epithelial cells, which is independent of the CagA protein in some *H. pylori* strains (Cendron et al., 2009; Fischer et al., 2001). To date, considerable attention has been given to the CagA effector protein, expanding our understanding of its function in the eukaryotic cell, however, the specific mechanism by which *H. pylori* has translocated CagA into the host gastric epithelial cell is largely unknown (Couturier et al., 2006).

The CagM (HP0537) locus has been found in a majority of clinical isolates, but much about its role has been unknown. Disruption of the cagM gene was reported to have an essential effect on CagA delivery and IL-8 induction phenotype (Fischer et al., 2001). Here, this paper reported the expression, purification and the immunogenicity of CagM. Meanwhile, we found that CagM localized to the bacterial inner and outer membrane. This research facilitates us to a further study of the protein-protein interaction between CagM and effector molecule CagA and a better understanding of the role of CagM in cagPAI and *H. pylori*.

MATERIALS AND METHODS

H. pylori strain and growth conditions

H. pylori strain 26695 (GenBank Accession No. NC_000915.1) was obtained from American type culture collection (ATCC). Frozen stocks of *H. pylori* strain 26695, which were stored at -80℃ were recovered on Skirrow´s medium supplemented with 10% glucose and 10% rabbit blood and the following antibiotics were added for selection: trimethoprim (5 μg/ml), vancomycin (10 μg/ml) and polymyxin B (2.5 IU/ml). Incubation of *H. pylori* strains was performed at 37℃ for 48 h in an anaerobic jar containing 10% CO_2, 5% O_2, and 85% N_2. Liquid cultures were grown in Skirrow´s medium supplemented with 5% glucose and 10% fetal bovine serum (FBS) in an anaerobic jar containing 10% CO_2, 5% O_2 and 85% N_2. Cultures were grown at 37℃ overnight, rotating at 160 rpm. The overnight culture was pelleted and stored at -80℃ for further use.

Cloning *of cagM* gene from *H. pylori* strain 26695

The cagM gene, based on the genome sequence of *H. pylori* strains 26695 was amplified by polymerase chain reaction (PCR) using Ex Taq DNA polymerase. The PCR primers were designed from the cagM gene sequence, with the forward primer cagM-P1 5'-GGATCCGCTAATGTGGAGCAGTTTG-3' containing a restriction site for *Bam*HI (underlined), and the reverse primer cagM-P2 5'-AAGCTTTTCAAAGGGATTATTCTTGGCAAC-3' containing a restriction site for *Hin*dШ (underlined). The PCR mixture was denatured at 94℃ for 40 s, annealed at 60℃ for 40 s, and extended at 72℃ for 1 min 10 s. A total of 25 cycles were performed. The resulting PCR product was purified and ligated into pMD19-T simple vector. Finally, this insert was transferred into pQE30 (Qiagen) prokaryotic expression vector via *Bam*HI and *Hind*Ш cleavage sites, generating the recombinant expression plasmid, designated pQE30-cagM.

Expression of recombinant CagM protein in *E.coli*

The recombinant plasmid, pQE30-cagM, was transformed into *E. coli* strain M15, and grown at 37℃ in 3 L LB medium containing ampicillin (100 mg/l) and Kanamycin (30 mg/l) to an OD_{600} of 0.6. At this point, the cells were shaken at 180 rpm at 37℃ temperature with 1 mM IPTG for an addition of 3.5 h. After bacterial cells were harvested by centrifugation at 5000 g for 8 min at 4℃, sedimentary pellet were treated and then separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the existence of the interest proteins. Subsequently, UVP gel scanner was applied to roughly estimate the expression level.

Purification of the fusion protein

After the bacteria had been harvested by centrifugation at 800 g for 10 min, the pellet was collected. The pellet was re-suspended in 300 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and disrupted under a pressure of 600 Mpa six times at a flow rate of 130 ml/min in ice-water bath using a high-pressure homogenizer (An Invernsys Group, Denmark), then followed by different centrifugation (750 g, 25 min; 12 000 g, 30 min) and the pellet containing the inclusion body was collected. The pellet was washed twice with 200 ml 1% Triton X-100, and twice with 200 ml 2 M urea solution, to remove the contaminants out of the insoluble precipitate. Inclusion body was re-suspended in sonication buffer (20 mM Tris-HCl, 8 M urea, pH 8.0), stirred slowly at 4℃ overnight, and centrifuged at 12 000 g for 20 min at 4℃. The supernatant was the dissolved inclusion body.

The dissolved inclusion body was loaded onto a Ni-nitrilotriacetic acid (Ni-NTA) agarose column, to which the 6×His-tagged recombinant protein would bind. The Ni-NTA agarose column was washed successively with wash buffer (20 mM Tris-HCl, 8 M urea, pH 8.0) and the 6xHis-tagged protein was eluted with the elution buffer (20 mM Tris-HCl, 250 mM imidazole, 250 mM NaCl and 8 M urea, pH 8.0). Elute was concentrated using an semipermeable membrane (the pore size below 10 kDa) and dialyzed in 0.1 M phosphate buffered saline (PBS) (pH 7.4) containing 4 M urea before subjected to animal assay. Protein concentration was determined by BCA protein assay kit. Molecular weight was estimated by both SDS-PAGE and mass spectrometry.

Antibody produced by immunizing with recombinant CagM protein

Rabbit polyclonal antibodies against the recombinant CagM protein were generated by immunizing Japanese large-ear rabbit with equal volumes of the antigen (1 mg) and Freund's complete or incomplete adjuvant on days 0, 21 and immunizing with 1 mg pure antigen on days 28 and 35. The antigen and Freund's adjuvant were mixed by vortexing until an emulsion was formed and injected subcutaneously into rabbits. The antiserum was collected by hemospasia from heart, and the antibody titer was evaluated by ELISA assay.

The antiserum was loaded into a MAbTrap G П Kit protein A sepharose high performance column. The protein A agarose column was washed successively with binding buffer (7.8 mM Na_2HPO_4 plus 12.2 mM NaH_2PO_4, pH 7.0) and elution buffer (100 mM glycine HCl, pH 2.7) successively and the IgG antibody protein was eluted with the elution buffer. After bound IgG was eluted, the eluate was immediately neutralized with 1 M Tris-HCl (pH 9.0) to pH 7.2 and the buffer was substituted by 0.1 M PBS (pH 7.2) with Hitrap desalting

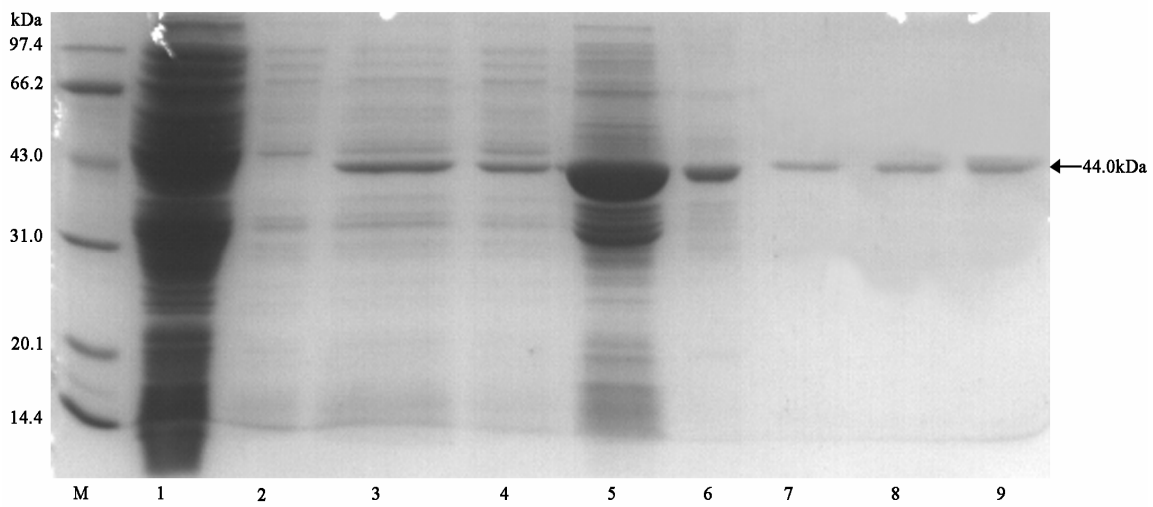

Figure 1. The expression and purification of induced fusion protein analyzed by SDS-PAGE. M, protein molecular weight marker; Lane1, whole cell lysate of *E. coli* M15 induced at 37℃; Lane 2, whole cell lysate from *E. coli* M15 containing pQE30-*cagM* before induction; Lane 3, whole cell lysate from IPTG-induced *E. coli* M15 containing pQE30-*cagM*; Lane 4, ultrasonic supernatant of recombinant strain induced by IPTG after 3.5 h; Lane 5, ultrasonic sediment of recombinant strain induced by IPTG after 3.5 h; Lane 6, cagM inclusion body washed with Tris-Cl buffer (pH 8.0) by adding various detergents.; Lane 7-9, the purified 6×Histagged cagM protein.

column. The purity of the IgG antibody was determined by SDS-PAGE and the activity and specificity of the purified antibody were tested by Western blotting analysis.

Fractionation of *H. pylori*

Bacterial fractionation was performed as described previously by Gauthier et al. (2003) and Couturier et al. (2006). *H. pylori* strain was grown overnight in Skirrow´s medium and the culture was harvested, washed in PBS (pH 7.4), and re-suspended in 1 ml of sonication buffer (10 mM Tris-HCl [pH 7] and protease inhibitors) and sonicated three times for 15 s each time (Fisher Sonicator, amplitude 2.5). Unbroken bacteria were removed by centrifugation (16,000 g, 5 min) at 4℃. The membrane pellet was re-suspended in 0.1 ml sonication buffer with 1% Triton X-100, which selectively solubilizes peri-plasmic proteins and centrifuged at 4℃ for 1 h. *N*-Lauroyl sarcosyl was used at 0.5% (wt/vol) for solubility of the inner membrane proteins with rotation at 4℃ for 1 h. The cytosolic fraction was recovered in 1 ml, the periplasmic fraction, the inner membrane and the outer membrane in 200 µl of each appropriate buffer. Equal percentages of each sample were analyzed by western blotting analysis.

SDS-PAGE and western blotting

SDS-PAGE and western blotting were performed as described previously (Cendron et al., 2009; Song et al., 2009). For visualization of proteins after SDS-PAGE, gels were stained with coomassie brilliant blue R250. For the development of immunoblots, PVDF filters were blocked with blocking buffer (Beyotime) and incubated with the respective antisera at a dilution of 1:1000. The membrane was washed six times with TBS containing 0.1% Tween-20 (TTBS) (pH 7.5). Horseradish peroxidaseconjugated anti-rabbit IgG was used at a dilution of 1:10 000 to visualize bound antibody.

RESULT AND DISCUSSION

Cloning and sequence analysis of cagM gene from *H. pylori* 26695

In the current work, using primers cagM-P1 and cagM-P2, a 1089 bp DNA fragment was successfully amplified and six continuous histidine residues to the N-terminal of the CagM were successfully added. The resultant sequence was cloned into the T-A cloning vector. The recombinant plasmid pMD19-T-*cagM* was digested by restriction endonucleases *Bam*HI and *Hin*dШ and the insert was subcloned into the digested pQE30 expression vector, then sequenced prior to submit to the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) for alignment. The alignment showed that the sequence was consistent to 26695 strain. The finally constructed pQE30-*cagM* was transformed into *E. coli* M15 for expression.

To analyze the divergence of *cagM* genes in 26695 strain and other *H. pylori* by Blast, the nucleotide and amino acid sequences identities were found more than 96%. By adding to the data of Blomstergrena et al. (2004) and Azuma et al. (2004), we can conclude that the sequence of cagM gene is conserved.

Protein purification and characterization

The CagM protein was overexpressed at 37℃ in *E. coli* strain M15 after 1 mM IPTG induction (Figure 1), and was found in two fractions, soluble and insoluble. There was

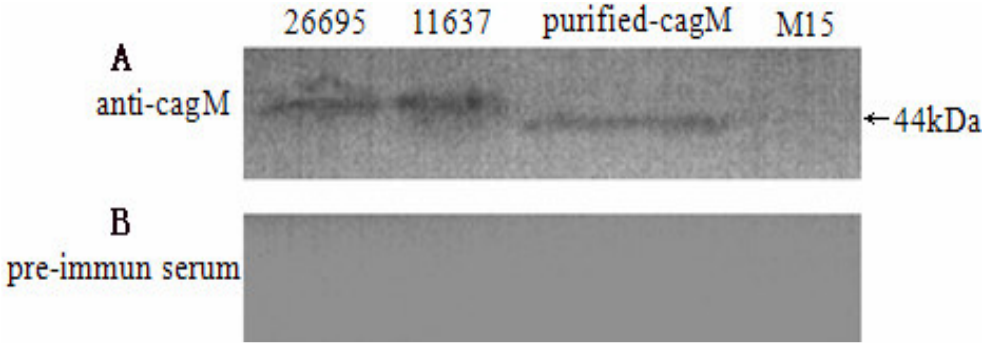

Figure 2. The activity and specificity of purified antibody of cagM. A: The whole lysis of 26695 and 11637 wild types and purified cagM protein can react strongly with anti-cagM antibody, and the *E. coli* M15 did not show the specific reaction with cagM antibody. B: No reactivity was observed in negative control of pre-immunized serum with 26695, 11637, purified cagM protein and *E. coli* M15.

more insoluble form than soluble protein (data not shown).

In order to remove the contaminated proteins in over-expressed *E. coli* strain M15 and facilicate CagM protein's purification, CagM inclusion body was washed with Tris-HCl buffer (pH 8.0) by adding various detergents. The effect of washing was perfect and the purity quotient of washed inclusion body reached above 85% (lane 6, Figure 1). Nickel metal-affinity resin column was used for single-step purification of 6×His-tagged CagM protein. The purity of 6×Histagged CagM protein was further examined by SDS-PAGE (lane 7 - 9, Figure 1), and a single band corresponding to the 44 kDa protein were observed with >95% purity. The result of it was to agree with the deduced molecular mass of CagM according to DNAssist software's prediction. The protein was quantified to be 2 mg/ml by BCA method as soon as possible after the purification.

Immunogenicity of CagM fusion protein

The IgG fraction against CagM was purified from rabbit antiserum by protein A Sepharose chromatography. The titer of the obtained IgG against CagM was determined by ELISA. The antibody at different dilutions (1000 to 128 000 fold) was reacted with an equal amount of the fusion protein (1 µg). The pre-immunized rabbit serum was used as the negative control. The antibody titer is defined as the highest dilution of serum at which the A490 ratio (A490 of postimmunization sera/A490 of preimmunization sera) is greater than 2.1. The values were the means ± SE (n = D3). The antibody titer was found to be more than 1:128 000. 4 M urea in purified CagM and it did not influence the effect of immunization.

The activity and specificity of the purified antibody was further tested by western blotting analysis with the purified anti-CagM antibody at a dilution of 1:1000. The result of western blotting with the antibody is shown in Figure 2. An expected ~44 kDa band of CagM detected in whole lysis of 26695 and 11637 wild types and purified protein showed the strong and specific reaction between them (lane 1 - 3, Figure 2A). In contrast, no reaction was observed in negative control with *E. coli* M15 (lane 4, Figure 2A). The same samples were also incubated with the pre-immunized serum but no reaction was observed (Figure 2B). The result indicated that *E. coli* strain M15 expressed 6x His-tagged CagM protein. The purified antibody can recognize the CagM with high activity and specificity, and serve as a good tool for further research the biofunctions related to CagM protein.

Subcellular localization of CagM

We used cell fractionation experiments to determine CagM's subcellular localization in *H. pylori*. This protocol has been well established for enteropathogenic *E. coli* and yielded a better fractionation than similar protocols used for *H. pylori* (Frankel et al., 1991; Fulkerson and Mobley, 2000). Through prediction software analysis, it was suggested that CagM is a 44 kDa protein with a pI of 9.29 (DNAssist) and has one strongly predicted transmembrane domain proximal to the N terminus of the protein (7 - 18 aa) (DAS [dense alignment sequence]; http://www.sbc.su.se/~miklos/DAS/) (Bendtsen et al., 2004; Nielson et al., 1997) and the transmembrane domain would suggest that a membrane localization is more likely for this protein than the cytoplasm (Couturier et al., 2006; Gardy et al., 2005).

Bacterial cultures were lysed using a French press and separated into cytosolic and membrane fractions following a procedure used to identify the membrane topology of the *H. pylori* CagM protein. As Figure 3A shows, the CagM antibody bound to the inner and outer membrane of *H. pylori* strain 26695. CagA is associated with the bacterial inner membrane (Couturier et al., 2006). The control of inner membrane antibody against the CagA, was strongly

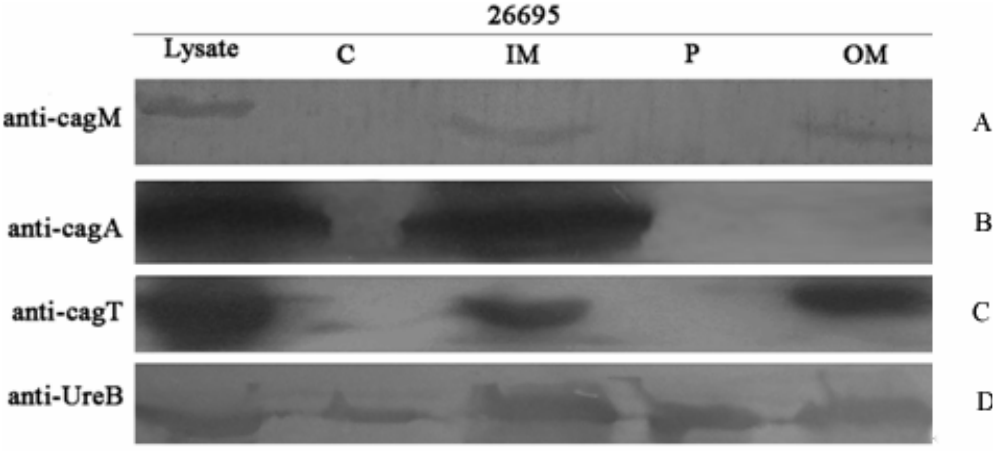

Figure 3. Bacterial fractionation. 26695 (wild type) was fractionated into four major fractions: cytoplasm (C), inner membrane (IM), periplasm (P), and outer membrane (OM). The cytoplasm was purified by ultracentrifugation, the periplasm by 1% Triton X-100 treatment, and inner and outer membranes by differential solubility in 0.5% (wt/vol) *N*-lauroyl sarcosyl. Equal percentages of samples were separated by 12% SDS-PAGE and transferred to PVDF membranes. Immunodetection of cagM was conducted by anti-cagM polyclonal sera. Immunoblots were probed with the control antibodies anti-cagA (inner membrane), anti-cagT (outer membrane) and anti-UreB (periplasm) to monitor the purity of the fractions.

reactive against the inner membrane fractions (Figure 3B). For the lack of the marker of the outer membrane, such as HopE, an outer membrane porin, we used CagT, which can be secret to the outer membrane and can be detected by immunofluorescence microscopy without permeabilizing as the outer membrane indicator (Figure 3C) (Rohde et al., 2003; Tanaka et al., 2003). UreB, spread in all parts of the cell, and its antibody was reactive against the cytoplasmic fraction, periplasmic protein, inner membrane and outer membrane fractions (Figure 3D). These results suggest that the separation of the bacterial fraction is efficient. This is contradicted to the conclusion of CagN (Bourzac et al., 2006) and CagF (Couturier et al., 2006). The prediction of association with the inner and outer membrane, combined with our results, and all confirm its inner and outer membrane localization.

Our data showed that, CagM partially associates with the inner membrane, where it is likely to be exposed to the periplasmic space. Such localization is supported by the strongly predicted N-terminal signal peptide of CagM. Given the signal peptide (SignalP v3.0; http://www.cbs.dtu.dk/services/SignalP/), a periplasmic localization would be expected.

Specific interaction between CagT and CagM from wild-type cells was found (Busler et al., 2006; Kutter et al., 2008). Moreover, a mutation in CagM caused the complete loss of CagT, which indicated a causal relationship between them (Fischer et al., 2001) and this information was consistent to ours (The result will be published in another paper which is processing). Combining these findings with our results, we may postulate that CagM plays an important role in assisting CagT's secretion, and further to maintains the stability of needle-like structure of *cag* PAI (Tanaka et al., 2003).

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